Experimental animal models for the study of moyamoya disease

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Moyamoya disease is a rare disorder of the cerebrovascular system affecting individuals in a bimodal age distribution and is characterized by progressive vascular stenosis of the bilateral supraclinoid internal carotid arteries with compensatory formation of collateral vessels at the base of the brain. Despite the disease's initial description in the literature in 1957, little progress has been made in the development of medical and surgical therapeutics due to, in no small part, the lack of effective experimental animal models. Currently, there is a poor understanding of the pathophysiological mechanisms behind the development of the moyamoya vasculopathies. Since the description of a genetic association between moyamoya disease, few studies have investigated the impact of genetic manipulation on the development of an animal model for experimentation. To date, no one model recapitulates the precise phenotype of the moyamoya vasculopathies, although development of an appropriate model would allow for an in-depth investigation into the pathophysiological mechanisms underlying the disease. In this review, the authors discuss the immunological, mechanical, and genetic methods used to develop moyamoya experimental models, as well as future perspectives.

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jury, intimal hyperplasia, and smooth muscle cell proliferation. Due to the reduced cerebral blood flow, a collateral network of blood vessels develops at the base of the brain, termed “moyamoya vessels.” However, from a histopathological perspective, in contrast to the appearance of the ICAs, the moyamoya collaterals do not exhibit the same inflammatory cellular components and, rather, exhibit features of accelerated vascular remodeling. Through familial analysis, the ring finger protein 213 (RNF213) gene located in the 17q25.3 region has been implicated in the pathogenesis of MMD, along with a variety of occlusive cerebrovascular diseases, and atherosclerotic intracranial major artery stenoses and occlusions.

Although strides have been made in understanding the pathophysiological mechanisms underlying MMD, the combination of disease heterogeneity and lack of animal models has led to stagnation in understanding the disease. An ideal MMD animal model would effectively replicate both the gradual, progressive vascular occlusion and the development of collateral basal vessels. A variety of methods have been developed in attempt to produce an effective MMD model, including mechanical occlusion or stenosis at various points within the cerebral vasculature, immune-mediated arterial injury, and genetic mechanisms. In this review, we will discuss the current state of experimental animal models for MMD and highlight potential directions for future work.

### Immunological Techniques for Moyamoya Model Development

Prior to the discovery of the genetic associations of MMD, early observations suggested that MMD may be a form of vasculitis with a predilection for the cerebral vasculature. Furthermore, a variety of early research indicated abnormal thrombogenesis and immune complex deposition within the vasculature of affected patients, ultimately lending to an “inflammatory and immunological” etiological proposition. In light of this theory, in 1992, Ezura et al. attempted to induce an MMD model in rabbits by implementing a serum sickness vasculitis model combined with intracisternal administration of antibodies or antigens. Rabbits were injected with heterologous serum via a combination of intravenous and intracisternal routes before being euthanized for analysis. Interestingly, rabbits that were exposed to only intravenous heterologous serum did not develop any features of cerebral arteritis. However, any rabbit exposed to intracisternal heterologous serum, regardless of intravenous exposure, exhibited transient features of cerebral arteritis with significant periarterial inflammatory cell infiltrate. These histopathological markers of arteritis were not present after 3 days and likely represented a transient local immune reaction to the intracisternal antigen deposition.

In 2003, Kamata et al. attempted to induce MMD in a feline model through a combined immunological-embolic method utilizing rod-shaped lactic acid–glycolic acid copolymer (LGA-50) and muramyl dipeptide (MDP) injected unilaterally into the ICA. This technique was successful in inducing an MDP-dependent immunological reaction within the intimal layer of the ICA. Similar to histological analysis of MMD-affected patients, the affected feline ICAs demonstrated mild intimal thickening with corresponding duplication of the internal elastic lamina within the terminal portions of the ICA, anterior cerebral artery (ACA), and middle cerebral artery (MCA). Interestingly, despite injection of the immuno-embolic agent unilaterally, histological changes of the intimal layers were present in the bilateral ICAs and its downstream tributaries. However, although a subset of the histological characteristics was replicated in the feline model, the development of moyamoya vessels at the base of the brain was not demonstrated with this technique. This may have been due to the difference in feline cerebral vasculature compared with human cerebral vasculature; feline brains receive a rich vascular supply from the external carotid artery. Therefore, the feline model did not exhibit severe cerebral hypoperfusion secondary to the unilateral ICA occlusion produced through this experimental technique.

In 2003, Terai et al. also utilized MDP to develop an experimental model for MMD in monkeys, which have a carotid artery system that is morphologically similar to that of humans. Lamination and reduplication of the internal elastic lamina of the intracranial arteries was seen, but neither stenosis of the carotid arteries nor development of collateral basal vasculature was observed. Although a number of groups have attempted to induce MMD in canine, monkey, and rat models after the study of Kamata et al., none were successful in replicating the full phenotype of MMD, and efforts since then have shifted in favor of mechanical and genetic methods of induction.

### Mechanical Techniques for Moyamoya Model Development

As patients with MMD exhibit features of chronic cerebral hypoperfusion (CCH), one of the most common methods of creating an experimental MMD animal model is through occlusion or stenosis of the common carotid arteries (CCAs) and ICAs at various points. Similar to other cognitive and vascular disorders, cerebral perfusion in MMD is diminished, and a variety of techniques have been developed both to recreate these hypoperfused states in animal models and to grade and treat patients with CCH. Given the underlying pathophysiological similarity between chronic vascular cognitive diseases and MMD, the experimental techniques have been adopted for use in MMD. A number of techniques have been developed to recreate this chronic hypoperfused state including bilateral CCA occlusion/stenosis in rats (also known as 2-vessel occlusion/stenosis), gradual bilateral CCA occlusion, and a 4- vessel occlusion model. An important consideration in the development of a CCH animal model is the tolerance to ischemic injury. The C57BL/6 mouse, a common experimental model, is extremely susceptible to cerebral ischemia and has demonstrated a high mortality rate following bilateral carotid artery occlusion, as demonstrated in 1997 by Yang et al. Furthermore, as a species, mice are more susceptible to ischemic injury and exhibit neuronal death within 30 minutes compared with the 60 to 120 minutes required to induce cell death in rats. From an anatomical perspec-
ative, a number of transgenic mice exhibit varying cerebrovascular organization and lack communicating arteries within the circle of Willis, severely reducing the animal's tolerance to infarcts.32.35 Despite the limitations of these animal models, they play a crucial role in the understanding of MMD, and many methods of inducing CCH have been trialed to date.

Carotid artery occlusion or stenosis remains the most common method of inducing CCH in rodents; however, it is important to note that a majority of these trials were conducted in the context of chronic vascular cognitive disease such as Alzheimer's disease or cerebral small vessel disease.16,23,26,36,37 Shibata et al. were able to successfully induce CCH in C57BL/6 mice through bilateral carotid artery stenosis using 0.18-mm-inner-diameter microcoils.36 Importantly, this model exhibited features of preserved gray matter structures and visual pathways due to maintenance of residual blood flow in the carotids and tributaries.

A factor complicating the development of an occlusion model is the abrupt interruption in blood flow when the carotid arteries are uni- or bilaterally occluded.13,23 A recent technique pioneered by Wang et al.20 in 2020, implemented a bilateral carotid artery stenotic mechanism in Wistar rats and demonstrated superior cognitive, vascular, and technical results in comparison with rats who underwent a bilateral carotid artery occlusion procedure. Importantly, cerebral blood flow remained persistently lower in the treatment groups in comparison with the control group for 14 days. While this model was developed with the goal of inducing a vascular cognitive impairment model, the reliable vascular reduction obtained in this study may prove promising for further studies evaluating this technique in the induction of an MMD model.23

Unfortunately, since many of these models have been developed for the purpose of vascular cognitive impairment replication, the histological and pathological vascular profiles of these models do not fit those of MMD precisely. MMS has a relatively similar anatomical profile to MMD; however, it is not associated with any genetic alterations. Because of this, in 2018 Roberts et al. developed a novel surgical model, termed internal carotid artery stenosis (ICAS) for MMS by placing microcoils in the proximal ICA in C57BL/6 mice.2 Following ICAS, decreased vessel diameter was observed in the ipsilateral ICA and ACA compared with the control mice. Of note, the experimental model was successful in mimicking the moyamoya-like vasculopathy seen in Suzuki stage I MMD. Although this study was conducted as an exploratory study with few animals and within a short timeframe, it provides the first experimental animal model created through surgical interventions specific for moyamoya vasculopathies.

**Genetic Techniques for Moyamoya Model Development**

The RNF213 gene, also known as the MMD susceptibility gene, encodes the ring finger domain, which, through E3 ubiquitin-protein ligase activity, modulates protein levels within mammalian tissues.38 By assisting the transfer of ubiquitin to a variety of heterologous substrates, RNF213 ultimately plays a crucial role in the regulation of cellular survival or death.14,38 Through familial whole genome analyses, a founder missense mutation in RNF213, p.R4859K, was found to be tightly associated with the development of MMD (OR 190.8, 95% CI 71.7–507.9).39 Its role in the pathogenesis of MMD has been a topic of interest, and recent studies have demonstrated an upregulation of the RNF213 gene in response to cerebral ischemia.14 In conditions of concurrent hypoxia and inflammation, commonly seen in patients with MMD, RNF213 mutations may predispose individuals to increased susceptibility to cerebral hypoxia secondary to aberrant and insufficient angiogenesis.18 In an effort to develop an experimental model through manipulation of the RNF213 gene, a number of studies have been conducted in a variety of animals; however, a paucity of information remains.

Initially established in zebrafish, two RNF213 genes, RNF213-a and RNF213-b, which produce amino acid sequences that are human RNF213 orthologs, were identified by Liu et al.10 Subsequently, RNF213 knockout zebrafish were produced and exhibited abnormal vascular development in the head, but relatively normal vasculature in the trunk. In a similar manner to the abnormal basal vasculature seen in MMD, knockout zebrafish specifically developed multiple aberrant vessels with irregular diameters originating from the inner optic circle and connecting to cranial vessels. This abnormal vascular development, specifically in the cranial regions, suggests that RNF213 plays a crucial, selective role in intracranial angiogenesis.

Given the promising results demonstrated in zebrafish, attempts at creating a murine MMD model through RNF213 knockout were attempted by Sonobe et al. in 2014.17 Through deletion of exon 32 of RNF213 using the Cre-lox system, homozygous knockout C57BL/6 mice were generated and observed. All knockout mice were observed for 64 weeks until they were euthanized, and radiological and histopathological analyses were conducted. In contrast to the effect of RNF213 knockout in zebrafish, radiological evaluation through MRA of RNF213 knockout mice did not reveal any gross malformations in the circle of Willis development, leptomeningeal anastomoses, or significant difference in vascular wall thickness compared with wild-type mice. However, RNF213 knockout mice that also underwent CCA ligation demonstrated significantly thinner intimal and medial layers compared with wild-type mice. In a corroborating study with RNF213 knockout mice, Kanoke et al. found no significant difference in circle of Willis anatomy or MRA findings.40 Ultimately, in contrast to knockout zebrafish, RNF213 knockout was not sufficient to induce MMD in mice, but additional mechanical stress may be necessary to induce an accurate MMD model.17,40

In a follow-up study to clarify the role of RNF213 knockout in inducing MMD, Sonobe et al. investigated the role of matrix metalloproteinase-9 (MMP-9) in RNF213 knockout mice following CCA ligation.41 RNF213 knockout mice demonstrated higher levels of MMP-9 and thinner vascular walls compared with wild-type mice, suggesting that the combination of insults may reflect early characteristic changes seen in MMD. From a cellular physiological standpoint, MMP-9 may be elevated in the
knockout mice due both to an increase in reactive oxygen species secondary to local ischemia following CCA ligation and also from local immunological interactions between the affected vascular wall and blood cells. The concept that an RNF213 mutation alone may not be sufficient to promote or induce MMD has led to discussion of various factors, such as local inflammatory factors, playing a role in the development of this disease. Vascular endothelial progenitor cells (EPCs) are crucial to the maintenance of the vascular bed by producing proangiogenic factors that not only promote survival of surrounding endothelial cells, but also assist in the function of smooth muscle progenitor cells (SMPCs). In MMD, immunohistochemical findings indicate both proliferation of endothelial cells and smooth muscle cells in the distal ICA, lending to the theory that the role of EPCs may be more involved than is currently understood. From a number of in vitro studies, EPCs have demonstrated impaired angiogenic potential in MMD and, therefore, are a potential target for in vivo model development. With regard to SMPCs derived from patients with MMD, Kang et al. demonstrated their aberrant activity in cell culture with abnormal gene expression and development of irregularly arranged and thickened tubes in cell culture. Due to the paucity of information regarding the biology of EPCs and SMPCs, a deeper understanding of the aberrant progenitor cells is necessary prior to the development of more accurate genetically produced MMD in vivo or in vitro experimental models.

Of note, the RNF213 knockout model has shown promise regarding development of the pathologic moyamoya vessels. In 2015, Ito et al. observed increased systemic angiogenesis in C57BL/6 RNF213 knockout mice following hind-limb ischemia. The authors were unable to identify a mechanism to explain this feature but speculated that given the increased MMP-9 concentrations found in patients with MMD, their murine model may have been that given the increased MMP-9 concentrations found in surrounding endothelial cells, but also assist in the function of smooth muscle progenitor cells (SMPCs). In MMD, immunohistochemical findings indicate both proliferation of endothelial cells and smooth muscle cells in the distal ICA, lending to the theory that the role of EPCs may be more involved than is currently understood. From a number of in vitro studies, EPCs have demonstrated impaired angiogenic potential in MMD and, therefore, are a potential target for in vivo model development. With regard to SMPCs derived from patients with MMD, Kang et al. demonstrated their aberrant activity in cell culture with abnormal gene expression and development of irregularly arranged and thickened tubes in cell culture. Due to the paucity of information regarding the biology of EPCs and SMPCs, a deeper understanding of the aberrant progenitor cells is necessary prior to the development of more accurate genetically produced MMD in vivo or in vitro experimental models.

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Discussion

The role of experimental animal models in the understanding of MMD cannot be overstated. Unfortunately, the limited availability of quality moyamoya models produces many challenges to the understanding of the pathophysiology behind the disease and, ultimately, to developing novel treatments for patients with MMD. Initial efforts to develop a model through immunological manipulation, unfortunately, did not result in an appropriate model but did lead to increased understanding of the disease, allowing for the development of more effective methods (Fig. 1). Animal models function as a translational facet for understanding the disease while acting as a segue for the development of various therapeutic agents. Through the development of genetic MMD models, recent studies have demonstrated the role of RNF213 not only in MMD but also in the pathogenesis of various systemic vasculopathies within the pulmonary and cardiac vasculature. This not only allows for improved treatment of these systemic vasculopathies but also aids in the development of genetic therapeutics targeted at correcting RNF213 mutations. As described previously, the development of MMS has been associated with the presence of autoimmune disease; however, the exact association between the two entities is not well understood. Using the ICAS model pioneered by Roberts et al., the combination of such models with a transgenic mouse that expresses autoimmune disease could provide a model to understand both the disease progression and potential treatment modalities for moyamoya vasculopathies.

From a surgical standpoint, current techniques focus on direct and indirect revascularization modalities, although both have ideal patient populations. A recent meta-analysis revealed the superiority of direct revascularization over indirect methods in regard to stroke risk; however, the most common complication following direct revascularization is hyperperfusion syndrome secondary to the increased cerebral blood flow. Management of hyperperfusion syndrome is characterized by adequate blood pressure and cerebral edema control; development of an adequate animal model would be invaluable for the experimentation of various operative methods. Indirect revascularization relies on the angiogenic potential of the grafted tissue. As discussed by Yu et al., understanding the biology of EPCs is crucial in improving the efficacy of indirect vascularized grafts.

There are a number of steps that could be implemented by the moyamoya research community that would aid in the development of an ideal experimental animal model. Standardizing the animal model development protocol would allow for more rapid progress. The Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines outline the “essential 10” items that aid researchers in improving the standards of animal model development from design conception through publication. A key feature lacking in prior moyamoya animal model studies is the randomization of animals exposed to the experimental group along with a blinded assessment of outcomes. When assessing for induction of moyamoya gross and histological characteristics, it is imperative that the assessing researcher is blinded to ensure reproducibility by subsequent investigators. Additionally, while some negative results have been reported in the literature regarding moyamoya experimental models, improved publication of these trials along with their methodologies would help guide future research in this field. Clinically, neurovascular imaging plays a central role in the diagnosis and treatment of MMD in humans. A more robust investigation of imaging
techniques in preclinical models may be instrumental in assessing the success of a novel animal model along with the radiological features of human MMD. Finally, from a basic science perspective, additional research in both the genetic and cellular biological fields would greatly enhance the limited understanding of the pathology underlying MMD.

As described in this review, a number of methods have been investigated in the development of an MMD model. Significant strides have since been made, and recent work has produced models that resemble moyamoya vasculopathies through surgical and genetic means, although neither method alone produces a perfect moyamoya replica. The development of the ICAS model in 2018 provides an easily replicable and implementable option for producing the cerebral hypoperfusion observed in patients with MMD. However, in the short study period, the ICAS model was unable to demonstrate the development of moyamoya vessels. Promising results for the development of the characteristic moyamoya vessels were seen in RNF213 knockout mice that exhibited increased systemic angiogenesis following exposure to transient hindlimb ischemia (Table 1).17,40,41 While we acknowledge the significant paucity of information within this realm, future work to combine the surgical and genetic approaches may be worthwhile to examine the effect of CCH in RNF213 knockout mice. The lack of information regarding the development of additional medical and surgical modalities highlights the need for a rapid increase in the number of preclinical and translational studies evaluating the biology of MMD.

Conclusions

Ultimately, the limited availability of a moyamoya ex-
TABLE 1. Overview of experimental moyamoya animal models

<table>
<thead>
<tr>
<th>Authors &amp; Year</th>
<th>Inducement Technique</th>
<th>Animal</th>
<th>Histological/Pathological Changes</th>
<th>Cerebral Hypoperfusion</th>
<th>Moyamoya Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ezura et al., 1992</td>
<td>Intraventricular serum sickness</td>
<td>Rabbit</td>
<td>Periarterial inflammatory cell infiltrate</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Kamata et al., 2003</td>
<td>Unilateral ICA injection of LGA-50 &amp; MDP</td>
<td>Cat</td>
<td>Intimal thickening &amp; duplication of internal elastic lamina in bilateral terminal ICA, ACA, &amp; MCA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Terai et al., 2003</td>
<td>MDP only</td>
<td>Monkey</td>
<td>Lamination &amp; duplication of internal elastic lamina w/o carotid artery stenosis</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Wang et al., 2020; Washida et al., 2019; Yang et al., 1997; Farkas et al., 2007</td>
<td>BCAO</td>
<td>C57BL/6 mouse</td>
<td>Neuronal death secondary to ischemic brain injury, high mortality</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Wang et al., 2020; Shibata et al., 2004</td>
<td>BCAS</td>
<td>C57BL/6 mouse &amp; Wistar rat</td>
<td>Persistent cerebral blood flow reduction</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Roberts et al., 2018</td>
<td>ICAS</td>
<td>C57BL/6 mouse</td>
<td>Decreased vessel diameter, Suzuki stage I</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Liu et al., 2011</td>
<td>RNF213 knockdown</td>
<td>Zebrafish</td>
<td>Abnormal cranial vascular development</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Sonobe et al., 2014</td>
<td>RNF213 knockdown</td>
<td>C57BL/6 mouse</td>
<td>None</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Ito et al., 2015; Sonobe et al., 2014; Kanoke et al., 2015</td>
<td>RNF213 knockdown &amp; CCA ligation</td>
<td>C57BL/6 mouse</td>
<td>Thinned intimal &amp; medial layers &amp; increased systemic angiogenesis</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

BCAO = bilateral carotid artery occlusion; BCAS = bilateral carotid artery stenosis.

Experimental animal model has stagnated and prevented the development of improved therapeutic options for patients with MMD. While the genetic method of producing an RNF213 knockout in mice has demonstrated positive results from the perspective of replicating the increased aberrant angiogenesis and histopathological changes seen in MMD, it is not yet capable of inducing the development of moyamoya vessels at the base of the brain. Similarly, surgical methods of reducing cerebral blood flow have shown promise in the replication of the cerebral hypoperfusion seen in MMD and also in reproducing the early pathological changes of the ICA seen in moyamoya patients. Despite their promising results, both aspects of MMD inducement have been sparsely tested and have been conducted as pilot studies. Further experimentation into the development of an adequate model is long overdue and would provide an invaluable resource for understanding MMD and the moyamoya vasculopathies, along with developing novel medical and surgical treatment options.

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**Disclosures**

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

**Author Contributions**

Conception and design: Ampie, Letchuman. Acquisition of data: Letchuman. Analysis and interpretation of data: Ampie, Letchuman, Mastorakos, Park. Drafting the article: Ampie, Letchuman, Mastorakos. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Ampie. Study supervision: Raper, Kellogg, Park.

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